(54) Title: MODIFIED FUNGAL CELLS AND METHOD FOR PRODUCING RECOMBINANT PRODUCTS

(57) Abstract

This invention relates to improved fungal cells and methods for producing recombinant products of improved quality and in high yields. More specifically, the present invention relates to fungal cells carrying specific modifications within their DNA sequences which cause them to exhibit at least a reduced capacity for O-glycosylating homologous and/or heterologous proteins, and the use of these cells as host cells to produce high yields of recombinant products.

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MODIFIED FUNGAL CELLS AND METHOD FOR PRODUCING RECOMBINANT PRODUCTS

This invention relates to improved fungal cells and methods for producing recombinant products of improved quality and in high yields. More specifically, the present invention relates to fungal cells carrying specific modifications within their DNA sequences which cause them to exhibit at least a reduced capacity for O-glycosylating homologous and/or heterologous proteins, and the use of these cells as host cells to produce high yields of recombinant products.

The development of recombinant DNA technology has made possible the production of foreign products in host cells in which exogenous DNA sequences coding for those products have been introduced. The advantage of this technology is that products can be produced in high yields, in highly purified form, with no risk of contamination such as viral contamination (AIDS, hepatitis B, etc.). These recombinant techniques have been widely used for the production of recombinant proteins in prokaryotic as well as eukaryotic host cells. Prokaryotic host cells include *E. coli* [Nagata et al., Nature <u>284</u> (1980), 316; EP 001 929], *Bacillus subtilis* [Saunders et al., J. Bacteriol. <u>169</u> (1987), 2917], *Streptomyces*, and *Corynebacterium* (EP 433 117). Eukaryotic host cells include plant cells, animal cells and fungal cells.

However, the large-scale production of recombinant products by these techniques is still limited, due to problems of expression efficiency of these exogenous DNA sequences, due also to vector instability and to intracellular degradation of the recombinant products by the host cell in which they are made. Concerning expression efficiency, efforts have been made to isolate strong promoters, leading to increased expression levels of exogenous DNA sequences, and therefore to increased production levels of recombinant products. Various systems have also been developed in order to increase the stability of the vectors within the host cells, the most frequently used of which consisting in the

insertion on the vector of an antibiotic resistance gene enabl-
ing recombinant host cells to survive and grow in a selective
medium. With respect to intracellular degradation, several
mutant cells lacking or having a reduced protease activity have
been disclosed, thereby limiting the capacity of said cells to
degrade recombinant products.

However, additional problems still limit large-scale production
and pharmaceutical use of recombinant products. One of these
arises from the fact that recombinantly produced products are
often different from their natural counterparts. For example,
bacterial host cells do not possess all the post-translational
mechanisms required for maturation of mammalian polypeptides.
Accordingly, said mammalian polypeptides produced in bacteria
are often immature or not correctly refolded. Furthermore, bac-
terial host cells generally introduce an additional N-terminal
methionine to the products.

Recombinant products produced in heterologous eukaryotic hosts
also usually differ from their naturally-occurring counterpart
in their glycosylation content. This may concern the presence
versus absence of any carbohydrate structure, the localization
of said carbohydrate structure on the product, as well as the
nature of the carbohydrate. More specifically, it has been shown
that yeast-derived recombinant products often bear additional
unnatural O-glycans compared to their natural counterpart. For
instance, it has been shown that, while human serum insulin-like
growth factor I (IGF-I) is not glycosylated, its recombinant
form produced in S. cerevisiae is O-glycosylated and, more pre-
cisely, O-mannosylated [Hard et al., FEBS Letters 248 (1989),
111]. In the same way, it has been shown that human platelet-
derived growth factor (PDGF) and human GM-CSF display unnatural
O-mannosyl structures when produced in S. cerevisiae [Biomedic.
Environ. Mass Spectrometry 19 (1990), 665; BIO/TECHNOLOGY 5
(1987), 831]. This abnormal O-glycosylation is the result of im-
portant differences between the glycosylation mechanisms of mam-
malian (human) cells and those of other eukaryotic cells, such
as yeasts. In this respect, it has been observed that O-glyco-

sylation in fungal cells (including yeasts and filamentous fungi) proceeds in a similar and unusual way so far not observed in any other organism.

The occurrence of this undesirable O-glycosylation on fungal-derived recombinant products constitutes an important drawback to this technology for the production of pharmaceuticals.

The first reason is that fungal-specific glycans may introduce new immunological determinants on a protein, and a glycoprotein with such unnatural carbohydrates may therefore be antigenic when administered to humans. In this respect, it is known for example that most humans have antibodies directed against N-linked yeast mannan chains [Feizi and Childs, Biochem. J. 245 (1987), 1].

Another reason is that proteins without appropriate carbohydrate structures may also have altered pharmacokinetic properties. It has been shown that carbohydrate structures of glycoproteins influence and participate in defining their *in vivo* clearance rate, which is essential in determining the efficacy of a pharmaceutical. More precisely, a mannose receptor has been identified on the surface of liver endothelial cells and resident macrophages which apparently represents a means for eliminating glycoproteins displaying mannose-type oligosaccharides [Stahl, Cell. Mol. Biol. 2 (1990), 317]. Therefore, the presence of unnatural, additional mannose structures on a protein may increase its clearance rate and thus decrease its plasma half life.

Still another reason is that biological activity of a glycoprotein has also been shown to vary with its carbohydrate content, location and nature. For example, it has been shown that glycosylation affects the biological properties of recombinant human EPO [Takeuchi et al., Proc. Natl. Acad. Sci. USA 86 (1989), 7819] and recombinant human tPA [Parekh et al., Biochemistry 28 (1989), 7644].

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For the reasons mentioned above, it is clear that the unnatural O-glycosylation of fungal-derived recombinant products can severely affect their immunological, biological and pharmacokinetic properties, and therefore may prevent their development for human therapeutic use.

The present invention solves the problem of abnormal O-glycosylation referred to above by providing modified fungal cells carrying genetic modification(s) within their DNA sequences which cause them to have at least a reduced capacity for O-glycosylating native or foreign proteins.

Applicant has found that it is possible to obtain genetically modified fungal cells having reduced capacity of O-glycosylation which are still viable and show good growth characteristics in industrial fermentation conditions. Unexpectedly, Applicant has also shown that said genetic modifications do not affect stability of these fungal cells when transformed with exogenous DNA. The modified fungal cells of the present invention can be utilized advantageously as host cells for the production of recombinant products of high quality, having reduced or no undesirable O-glycans.

One object of the present invention is a fungal cell carrying genetic modification(s) within its DNA sequences which cause it to have at least a reduced capacity of O-glycosylation.

The fungal cell of the present invention can be chosen from filamentous fungi and yeasts. Exemplary genera of filamentous fungi contemplated by the present invention are *Aspergillus*, *Trichoderma*, *Mucor*, *Neurospora*, *Fusarium* and the like. Exemplary genera of yeasts include *Kluyveromyces*, *Saccharomyces*, *Pichia*, *Hansenula*, *Candida*, *Schizosaccharomyces* and the like. More preferred genera are those selected from the group consisting of *Kluyveromyces*, *Saccharomyces*, *Pichia*, *Hansenula* and *Candida*, and, even more preferred, from *Kluyveromyces* and *Saccharomyces*. Exemplary strains of *Kluyveromyces* which constitute preferred embodiments of this invention include *K. lactis*, *K. fragilis*, *K.*

waltii, K. drosophilarum and the like. The preferred strain of *Saccharomyces is S. cerevisiae.*

In the meaning of the present invention, genetic modification preferably means any suppression, substitution, deletion or addition of one or more bases or of a fragment of the fungal cell DNA sequences. Such genetic modifications may be obtained *in vitro* (directly on isolated DNA) or *in situ*, for example by genetic engineering techniques or by exposing the fungal cells to mutagenic agents. Mutagenic agents include for example physical agents such as energetic rays (X-rays, χ-rays, UV, etc.) or chemical agents capable of reacting with different functional groups of DNA, such as alkylating agents (EMS, NQO, etc.) bisalkylating agents, intercalating agents, etc. Genetic modifications may also be obtained by genetic disruption, for example according to the method disclosed by Rothstein et al. [Meth. Enzymol. 194 (1991), 281-301].

According to this method, part or all of a gene is replaced, through homologous recombination, by an *in vitro* modified version.

Genetic modifications can also be obtained by any mutational insertion on DNA sequences, such as transposons, phages, etc.

In addition, it is known that certain modifications such as point mutations can be reversed or attenuated by cellular mechanisms. Such modifications may not provide the most useful forms of modified fungal cells of this invention since their phenotypical properties may not be very stable. The present invention also provides a process for preparing modified fungal cells in which the modifications (and therefore the phenotypical properties) are stable during segregation and/or non-reverting and/or non-leaky. Such modified fungal cells are particularly advantageous as hosts for the production of recombinant products.

Accordingly, a preferred embodiment of the invention is a fungal cell carrying genetic modification(s) which are stable during

segregation and/or non-reverting and/or non-leaky. These modifications are generally obtained by deletion(s) or disruption(s).

The genetic modification(s) carried by the fungal cells of the invention can be located either in a coding region of the DNA sequences of the cell or in a region responsible for or involved in the expression and/or transcriptional regulation of a gene. More particularly, said modification(s) will generally affect the coding region or the region responsible for or involved in the expression and/or the transcriptional regulation of one or more genes whose expression products are enzymes of the O-glycosylation pathway.

The reduced capacity of the fungal cells of the invention to O-glycosylate proteins may therefore result from the production of inactive enzymes due to structural and/or conformational changes, from the production of enzymes having altered biological properties, from the absence of production of said enzymes, or from the production of said enzymes at low levels.

The fungal cell O-glycosylation pathway involves attachment of a first mannosyl residue to the hydroxyl group of seryl and/or threonyl amino acids of proteins or peptides, and then the extension to O-linked di- and oligosaccharides by subsequent addition of mannosyl residues. The first mannosyl residue is transferred from dolichol monophosphate mannose (Dol-P-Man) to the protein in the endoplasmic reticulum, and the additional mannosyl residues are transferred from GPD-Man in the Golgi. In contrast, higher eukaryotic (non-fungal) cells O-glycosylate following a different mechanism, in that the initial step is the covalent attachment of N-acetyl-galactosamine to seryl or threonyl amino acids, no lipid-coupled oligosaccharide donor is involved in this first reaction, the initial step occurs in the Golgi, the structures of carbohydrates are different, etc.

In a preferred embodiment of the invention, the modified fungal cells carry genetic modification(s) in at least one gene whose

expression product is involved in the attachment of a mannosyl residue to the hydroxyl group of seryl or threonyl amino acids.

In a more preferred embodiment of the invention, the modified fungal cells carry genetic modification(s) in at least one gene whose expression product is involved in the transfer of a mannosyl residue from the Dol-P-Man precursor to the hydroxyl group of seryl or threonyl amino acids.

Still more preferably, one of these genes is the gene encoding the Dol-P-Man:Protein (Ser/Thr) Mannosyl Transferase [DPM2 - also designated PMT1] whose sequence is represented in Figure 4, or any homologous gene encoding the same activity as defined below.

In addition to modification(s) in one gene involved in the attachment of mannosyl residues to the hydroxyl group of seryl or threonyl amino acids, fungal cells of the invention may also carry modification(s) in the genes involved in subsequent additions of mannosyl residues leading to di- or oligosaccharides, or in the synthesis of the mannosyl residues donnor (Dol-P-Man).

Specific examples of such fungal cells are disclosed in the examples.

Another object of the invention resides in a fungal cell as disclosed above in which an exogenous DNA sequence has been introduced.

In the meaning of the present invention, the term exogenous DNA sequence includes any DNA sequence comprising one or more genes encoding a desired protein to be expressed and/or secreted in said cell. Such a DNA sequence may be a complementary DNA sequence (cDNA), an artificial DNA sequence, a genomic DNA sequence, a hybrid DNA sequence or a synthetic or semi-synthetic DNA sequence, included in an expression cassette enabling synthesis in the fungal cells of said proteins. The expression cassette preferably comprises a transcription and translation

initiation region joined to the 5' end of the sequence encoding said desired protein(s) so as to direct, and optionally regulate, the transcription and translation of said sequence. The choice of these regions may vary according to the fungal cell used. Generally, these sequences are chosen from promoters and/or terminators derived from fungal cell genes, and, when expression in yeast hosts is sought, from yeast genes. Of special interest are certain promoter and/or terminator regions derived from glycolytic genes of fungal cells such as, for yeasts, the genes encoding phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate dehydrogenase (GDP), enolases (ENO) or alcohol dehydrogenases (ADH), and for filamentous fungi, the genes encoding triose phosphate isomerase (tpi). The promoter and/or terminator regions may also derive from other strongly expressed genes such as, for yeasts, the lactase gene (LAC4), the acid phosphatase gene (PHO5), the alcohol oxidase gene (AOX) or the methanol oxidase gene (MOX), and, for filamentous fungi, the cellobiohydrolase gene (CBHI), the alcohol dehydrogenase gene (alcA, alcC), the glucoamylase gene (GAM) or the acetamidase gene (amds), and the like. These transcription and translation initiation regions may be further modified, e.g. by in vitro mutagenesis, by introduction of additional control elements or synthetic sequences, or by deletions. For example, transcription-regulating elements, such as the so-called UAS, originating from another promoter may be used to construct hybrid promoters which enable the growth phase of the fungal cell culture to be separated from the phase of expression of the desired protein(s) encoding sequence(s). A transcription and translation termination region functional in the intended fungal cell may also be positioned at the 3' end of the coding sequence. In addition, at the N-terminus of the protein sequence, a signal peptide (pre-sequence) may be introduced so as to direct the nascent protein to the secretory pathway of the fungal cell used. This pre-sequence may correspond to the natural pre-sequence of the protein if this protein is naturally secreted, or it may be of another origin, e.g. obtained from another gene, or even artificial.

Preferably, the exogenous DNA sequence is part of a vector,
which may either replicate autonomously in the fungal cell used
or integrate into its own DNA sequences (chromosome). Autono-
mously replicating vectors may contain autonomously replicating
sequences derived from the chromosomal DNA of the fungal cell
(ARS) or from naturally-occurring fungal cell plasmids such as
pGKl-1 [de Louvencourt et al., J. Bacteriol. 154 (1982), 737],
pKD1 (EP 241 435), 2μm plasmid (Broach, Cell 28 (1982), 203-204)
and the like. Integrating vectors usually contain sequences ho-
mologous to regions of the fungal cell chromosome which, after
being introduced into said cell, enable integration through in
vivo recombination. In a specific embodiment of the invention,
said homologous sequences correspond to the region of the chro-
mosome to be modified in the fungal cell, enabling a one-step
modification-integration mechanism. Integration may also occur
through non-homologous recombination.

The exogenous DNA sequence can be introduced into the fungal
cell by any technique known in the art, and, for example, by re-
combinant DNA techniques, genetic crossings, protoplast fusions,
etc. Concerning recombinant DNA techniques, transformation,
electroporation, or any other technique disclosed in the litera-
ture may be used. More specifically, when the fungal cell is a
yeast cell, the transformation may be performed according to the
methods of Ito et al., [J. Bacteriol. 153 (1983), 163], Durrens
et al. [Curr. Genet. 18 (1990), 7] or following the method
disclosed in EP 361 991. Electroporation can be performed ac-
cording to Karube et al. [FEBS Letters 82 (1985), 90].

The fungal cells of the present invention can be advantageously
utilized as host cells for the production of recombinant pro-
ducts such as heterologous proteins having pharmaceutical and/or
agro-foodstuff interest. The fungal cells of this invention are
particularly advantageous since they enable the production
and/or secretion of high quality products, and since their gene-
tic modifications do not affect the mitotic or genetic stability
of said products' expression vectors. The cells of this inven-
tion are more particularly suitable for the production of pro-

teins having human therapeutic uses and which are susceptible to O-glycosylation by the host cell.

Accordingly, a further object of this invention resides in a process for the production of recombinant products wherein a fungal cell as defined above is cultivated in conditions in which the exogenous DNA sequence is expressed and the product is recovered. In a preferred embodiment, said product is secreted into the culture medium. In another preferred embodiment, said product is susceptible to O-glycosylation by the host cell.

The following proteins are cited as examples of heterologous proteins which can be prepared with the fungal cells of the present invention: enzymes (such as superoxide dismutase, catalase, amylases, lipases, amidases, chymosine, etc., or any fragment or derivative thereof), blood derivatives (such as human serum-albumin, alpha- or beta-globin, factor VIII, factor IX, van Willebrand factor, fibronectin, alpha-1 antitrypsin, etc., or any fragment or derivative thereof), insulin and its variants, lymphokines [such as interleukins, interferons, colony stimulating factors (G-CSF, GM-CSF, M-CSF...), TNF, TRF, etc., or any fragment or derivative thereof], growth factors (such as growth hormone, erythropoietin, FGF, EGF, PDGF, TGF, etc., or any fragment or derivative thereof), apolipoproteins, antigenic polypeptides for the preparation of vaccines (hepatitis, cytomegalovirus, Eppstein-Barr, herpes, etc.), or any fusion polypeptide such as, for example, fusions comprising an active moiety linked to a stabilizing moiety.

Another object of the invention resides in a DNA fragment encoding an enzyme involved in the attachment of mannosyl residues to the hydroxyl group of seryl or threonyl amino acids of proteins. Applicant has provided DNA fragments encoding such enzymes for the first time. More preferably, said DNA fragment comprises the Dol-P-Man:Protein (Ser/Thr) mannosyltransferase gene whose sequence is represented in Figure 4, any homologous gene, derivative or fragment thereof.

In the meaning of the present invention, homologous gene means
any other gene of any fungal cell encoding an enzyme having the
required activity. Said other genes may be obtained, for exam-
ple, by complementation of a mutant fungal cell deficient in
said activity with DNA prepared from a fungal cell capable of
said activity, selection of the transformants having recovered
the activity, and isolating their inserted DNA sequence. These
other genes may also be isolated from DNA libraries by hybridi-
zation with probe(s) (including PCR primers) comprising all or
part of the sequence presented in Figure 4. In this respect, it
is also an object of this invention to use the DNA fragments
provided, or any part thereof, as hybridization probe(s) or for
the complementation of mutant phenotypes, for the obtention of
homologous genes of fungal cells.

The term derivative means any other DNA fragment prepared by any
genetic and/or chemical modification(s) of the genes mentioned
above. Said genetic and/or chemical modification(s) may be any
suppression, substitution, deletion or addition of one or more
bases or of a region of said genes, leading either to an in-
creased enzyme activity or to the same activity level, or to a
decreased or null enzyme activity upon transformation in a fun-
gal host cell.

LEGEND OF THE FIGURES

Figure 1:   Restriction map of plasmids pDM3, pMT4 and pMT1.
Figure 2:   Subcloning of plasmid pDM3.
Figure 3:   Strategy of sequencing of the PMT1 gene.
Figure 4a: Nucleotide sequence of the PMT1 gene (SEQ ID N°1).
Figure 4b: Amino acid sequence of the PMT1 gene (SEQ ID N°2).
Figure 5:   Construction and restriction map of pMT1.1/URA3.
Figure 6:   O-glycosylation activity of *S. cerevisiae* WT (panel
            A) and MT (panel B).
Figure 7:   Partial nucleotide sequence of the *K. lactis* PMT1
            gene (SEQ ID No. 3).
Figure 8:   Nucleotide (Panel A) and predicted amino acid (Panel
            B) sequence comparison between the *S. cerevisiae* PMT1
            gene (upper sequences) and the *K. lactis* homolog
            (lower sequences) isolated by PCR amplification of *K.
            lactis* genomic DNA. Dots represent sequence identity,

question marks indicate sequence ambiguity. The
nucleotide sequence complementary to the primer
Sq3910 is underlined.

EXAMPLES

Example 1: Isolation of a highly purified mannosyltransferase from *S. cerevisiae* and generation of peptides

The mannosyltransferase activity was solubilized from total
yeast membranes and purified on hydroxylapatite according to
Strahl-Bolsinger and Tanner (Eur. J. Biochem. 196 (1991), 185).
The protein then had to be further enriched by $(NH_4)_2SO_4$ preci-
pitation before additional purification was performed via affi-
nity chromatography. The eluted material was then separated on
SDS/PAGE. The resulting 92 kDa band was cut out of the gel.
Trypsin digestion (in the gel) yielded several non-overlapping
peptides, enabling designing of probes.

E.1.1. $(NH_4)_2SO_4$ precipitation

100 ml of fractions of the hydroxylapatite column containing
mannosyltransferase activity was mixed with $(NH_4)_2SO_4$ up to a
final concentration of 30% (w/v) and stirred gently for 1 h in
an ice/salt bath. The mixture was centrifuged for 30' (8000 x
g). The resulting pellet was resuspended in 8 ml AB-buffer (10
mM Tris/HCl, pH 7.5, 15% glycerol (vol%), 0.1% lubrol (vol%),
150 mM NaCl) and dialyzed for 1 h against the same buffer.
Storage: -20°C.

E.1.2. Affinity chromatography

E.1.2.1. Preparation of the affinity chromatography column

0.5 g freeze-dried powder of Protein A-Sepharose Cl 4B was swol-
len in 10 ml 100 mM NaPi, pH 7.0, for 15' and washed on a sin-
tered glass filter (G3) with 200 ml of the same buffer. Protein
A-Sepharose Cl 4B was equilibrated in 100 mM NaPi, pH 7.0. About
3 to 6 ml anti-mannosyltransferase serum was dialyzed for 2 h
against 1 l NaPi (100 mM), pH 7.0. The dialyzed serum was incu-
bated with the column material for 16 h at 4°C. The serum was

removed using a sintered glass filter (G3). The column material was washed twice with 10 ml 100 mM NaPi, pH 8.5, and resuspended in 50 ml of the same buffer.

For covalent coupling 0.75 mg/ml dimethylsuberimidate was added. The pH was adjusted to pH 8.5 by adding 5-6 drops of 1 M NaOH. The material was incubated for 1 h at RT. For a second time, dimethylsuberimidate was added and the pH adjusted to pH 8.5 with 1 M NaOH. The column material was washed serially on a sintered glass filter (G3) with:

a) 50 ml 100 mM NaPi, pH 8.0
b) 25 ml 100 mM NaPi, pH 8.0, 3 M ammonium rhodanide
c) 100 ml 100 mM NaPi, pH 8.0

The material was washed and equilibrated in AB-buffer.

E.1.2.2. Purification of the 92 kDa protein

8 ml of the $(NH_4)_2SO_4$ precipitated and dialyzed protein (E.1.1.) was incubated with the affinity column material (E.1.2.1) for 16 h at 4°C with gentle shaking. A column (2 cm x 0.5 cm) was filled and washed with 15 ml AB-buffer. The column was eluted with 100 mM glycine/HCl pH 3.0, 0.05% lubrol (vol%), 15% glycerol (vol%). Fractions of 0.9 ml were collected and neutralized immediately with 1 M Tris (15 μl/0.9 ml fraction).

To detect the 92 kDa protein, 40 μl of each eluted fraction was analyzed by SDS/PAGE and Western-blot analysis as described (Strahl-Bolsinger and Tanner, 1991). The 92 kDa protein containing fractions (fraction 2-6) were pooled and concentrated to 100 μl via microconcentrators (Centricon/Amicon) by centrifugation at 5000 x g. 0.9 ml 98% EtOH were added and the protein precipitated for 16 h at -20°C. The precipitated protein was pelleted by centrifugation for 30' at 10,000 x g.

E.1.3. SDS-PAGE

The precipitated protein (E.1.2.2) was resuspended in 150 μl SDS-sample buffer (0.07 M Na_2CO_3, 0.07% ß-EtSH, 2% SDS, 12% Saccharose, 0.07% bromphenolblue). SDS-gel electrophoresis according to Lämmli and Favre [J. Mol. Biol. 80 (1973), 575] was carried out at 50-70 V using the BIORAD-Mini-Protean cell. Protein standards: HMW-Standards/Gibco BRL.

Protein was detected by staining with 0.05% Coomassie R250 (w/v), 25% isopropanol (vol%), 10% acetic acid (vol%) and destaining in 7.5% acetic acid (vol%).

E.1.4 Trypsin digestion and designing of oligonucleotides

After SDS-PAGE (E.1.3.) the 92 kDa protein band was cut out (about 10 μg of protein). The gel fragment was cut into small pieces and shaken three times for 30' in 5 ml 50% methanol/10% acetic acid and one time for 30' in 5 ml 50% methanol. The gel was lyophylized for 3 h. Trypsin digestion was carried out in 0.3 ml 0.2 M ammoniumhydrogen carbonate/2μg trypsin for 16 h at 37°C. Supernatant was removed. Elution of the peptides was done three times for 1 h at 37°C in 0.2 ml 0.2 M ammoniumhydrogen carbonate and one time for 1 h at 37°C in 0.2 ml 0.2 M ammonium-hydrogen carbonate/30% acetonitrile. The eluted material was pooled, lyophylized and resolved in 0.2 ml 1M guanidinium hydro-chloride/50 mM Tris/HCl, pH 7.5. Peptides were separated using a reverse phase RP18 column equilibrated in 0.13% TFA. Peptides were eluted by acetonitrile (0-70%). Up to 40 different peptide peaks could be detected. Five of the main peaks were sequenced via automated sequence analysis according to Edman (G. Allen in: Sequencing of proteins and peptides, Laboratory Techniques in Biochem. and Mol. Biol. 9 ed.: Burdon, R.H. & Knippenberg, P.H.; Elsevier (1989)). Among the sequences thereby obtained, three were suitable for designing oligonucleotides, which are presented in Table 1, below.

Table 1

Peak	Peptide Sequence
15	I S Y K P A S F I S K
23	E V S P Y G Y S G F D G D A
34	N L V E P H V Y E S

On the basis of these sequences, oligonucleotides A-C were chemically synthesized, using the codon usage of *S. cerevisiae* (Guthrie and Abelson in: The molecular biology of the yeast Saccharomyces; eds: J.N. Strathern, E.W. Jones, J.R. Broach (1982)). Oligonucleotides A-C have the following character-istics:

- Oligonucleotide A:

    peak: 23

    amino acid sequence: G F D G D A

    Oligodeoxynucleotide: 5'-G^T/$_C$GTCACCGTCGAANCC-3'

    8-fold degenerated, coding strand, 17 nucleotides

- Oligonucleotide B:

    Peak: 34

    Amino acid sequence: E P H V Y E

    DNA sequence: 5'-^{C}/$_T$TCGTAGAC^G/$_A$TG^A/$_T$GG^T/$_C$TC-3'

    16-fold degenerated, coding strand, 18 nucleotides

- Oligonucleotide C:

    Peak: 15

    Amino acid sequence: I S Y K P A S F I S K

    DNA sequence: 5'-ATTTC^T/$_A$TA^T/$_C$AA^A/$_G$CC^A/$_T$GCTTC^T/$_A$

        TT^T/$_A$AAA-3'

    128-fold degenerated, coding strand, 33 nucleotides

Example 2: Screening of a plasmid library of yeast genomic DNA

The chemically synthesized oligodeoxynucleotides A-C (E.1.4.)
were used to screen the plasmid library of yeast genomic DNA
pCS19 (Sengstag and Hinnen, Nucl. Acids Res. 15 (1987), 233).
This library was prepared by partial digestion of yeast genomic
DNA with Sau3A, and cloning into the BclI restriction site of
the vector pCS19.

E.2.1. Labeling of Oligodeoxynucleotides

The oligonucleotides A-C were labeled by kinase reaction, car-
ried out according to Maniatis et al (T. Maniatis, J. Sambrook,
E.F. Fritsch (1989), Molecular cloning: A Laboratory manual,
C.S.H. Press). 40 pmol Oligodeoxynucleotide were labeled using
50 μCi [γ-^{32}P]-ATP. Free radioactive nucleotides were removed
using "NUC Trap Push columns" (Stratagene) according to the in-
struction manual of the producer.

E.2.2. Screening of the library

The DNA-library (4992 different single colonies) was transferred
from microtiterplates to nitrocellulose. Colony hybridization
was performed according to Grunstein and Hogness (PNAS 72
(1975), 3961) in the following conditions:

- Prehybridization: Filters were incubated at 44°C in 200 ml 5
x Denhardt's, 6 x NET, 0.1% SDS (w/v), 0.1 mg/ml salmon sperm
DNA, for at least 4 h (5 x Denhardt's: 0.1% ficoll, 0.1% poly-
vinylpyrrolidone, 0.1% BSA; 6 x NET: 0.9 M NaCl, 90 mM Tris-HCl,
pH 8.3, 6 mM EDTA, pH 8.0).

- Hybridization: Filters were incubated at 44°C in 100 ml 5 x
Denhardt's, 6 x NET, 0.1% SDS (w/v), 0.1 mg/ml salmon sperm DNA,
labeled oligodeoxynucleotides A and B (40 pmol each). Hybridiza-
tion was performed for 16 h.

- Washing conditions: Filters were washed three times in 50 ml
6 x SSC, 0.1% SDS (w/v) at 0°C for 15'.

To detect positive colonies, the filters were exposed to X-ray
films for 16 h, -70°C. Under these conditions, 12 positively re-
acting clones could be identified.

Example 3: Southern Analysis of the 12 positive clones

The 12 positive clones were analyzed in Southern blots using
three different oligodeoxynucleotides. This analysis led to the
identification of one positive clone reacting with all three
oligonucleotides. This clone was called pDM3.

The 12 positive clones were grown in 5 ml LB medium supplemented
with ampicillin and their DNA was isolated according to the me-
thod of Birnbaum and Doly (Nucl. Acid. Res. 7, (1979), 1513).

1/10 of each isolated plasmid DNA (plasmids: pDM1-pDM12) was di-
gested with the restriction enzymes EcoRI-XhoI (5U each), 1 x
"one for all" buffer (Pharmacia) in a total volume of 20 μl for

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1 h at 37°C. DNA fragments were separated on a 1% agarose gel and blotted to nitrocellulose according to Maniatis et al. (loc. cit.). Southern analysis was performed using oligo A and B using the same conditions as described for the library screen. The hybridization temperature for oligo A was 48°C, for oligo B 42°C. Clones 1, 2, 3, 5, 6, 7 and 11 reacted positively with both oligodeoxynucleotides. These seven clones were further analyzed by Southern blot analyses. Three identical blots were therefore prepared, in which the DNA of clones 1, 2, 3, 5, 6, 7 and 11 was digested with EcoRI-XhoI and blotted to nitrocellulose as described. Blots 1, 2 and 3 were prehybridized in 20 ml 5 x Denhardt's, 6 x NET, 0.1% SDS (w/v), 0.1 mg/ml salmon sperm DNA at 50°C for 4 h. Each blot was then hybridized in 10 ml 5 x Denhardt's, 6 x NET, 0.1% SDS (w/v), 0.1 mg/ml salmon sperm DNA, 40 pmol labeled oligonucleotides for 16 h. The hybridization temperature is indicated in Table 2, below. Washing was performed for 10' at each temperature in 50 ml 2 x SSC, 0.1% SDS (w/v).

Table 2

Blot	Hybridization with oligo	Hybridization temperature	Washing conditions	Positive reacting clones
Blot 1	A	25	2x10'/25°C 1x10'/35°C	1,2,3,5,6,7,11
Blot 2	B	25	2x10'/25°C 1x10'/35°C	3, 5
Blot 3	C	45	2x10'/45°C 1x10'/55°C	3

Clone 3 was the only clone reacting with oligo A, B and C. The clone was called pDM3 and further analyzed.

Example 4: Analysis of pDM3

E.4.1. Methods

E.4.1.1 Digestion with restriction endonucleases

Analytic digestion with endonucleases was performed in 1 x "one for all" buffer (Pharmacia), 0.2 - 0.5 μg of DNA, 1-5 U restriction enzyme in a total volume of 20 μl for 1 h at 37°C.

Preparative digestion was performed in a total volume of 40-80 μl with 1-10 μg of DNA, 5-20 μl restriction enzyme, 1 x "one for all" buffer for 2 h at 37°C.

E.4.1.2. DNA-gelelectrophoresis

Separation of DNA fragments was performed according to Maniatis et al. (loc. cit.).

E.4.1.3. Isolation of DNA fragments

After separation, DNA fragments were isolated using the "Gene-clean kit" (Stratagene) according to the instruction manual of the producer.

E.4.1.4. Treatment with alkaline phosphatase

DNA fragments were dephosphorylated with alkaline phosphatase according to Maniatis et al. (loc. cit.).

E.4.1.5. Ligation

DNA fragments were ligated in 1 x T4-ligation buffer (50 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP) with 1 U T4-DNA ligase (total volume 10-15 μl). Molar DNA ratio of vector: insert was 1:4 or 1:8. The absolute amount of DNA was 20-50 ng. Incubation time: 16 h at 14°C or 5 h at 25°C.

E.4.1.6. Transformation of *E. coli*

Competent *E. coli* DH5α cells were prepared according to Hanahan (J. Mol. Biol. 166 (1983), 557). Transformation was carried out as described by Maniatis et al. (loc. cit.).

E.4.1.7. Preparation of DNA

Plasmid DNA was prepared according to Birnbaum and Doly (loc. cit.).

E.4.1.8. Southern blot analysis

Southern blot analysis was performed using the same condition as described in E.3.

E.4.1.9. DNA sequence analysis

DNA sequencing was done according to the method of Sanger et al. (PNAS 74 (1977), 5463). Only plasmid DNA was sequenced. T7-DNA polymerase sequencing kit (Pharmacia) was used; the radioactive nucleotide was $[\alpha-^{35}S]$-dATP (spec. act. 600 Ci/mmol).

E.4.2. Identification of the ORF

This example discloses a restriction analysis of pDM3, the identification of different DNA fragments recognized by oligonucleotides A, B or C and their subcloning. Sequencing of these subclones enabled identification of an ORF.

E.4.2.1. Subcloning of pDM3 DNA fragments hybridizing with oligo A, B or C.

pDM3 DNA was digested with EcoRI, XhoI and EcoRI-XhoI. Southern blot analysis was performed using oligo A, B or C as a target.

Oligo A recognizes a 3.0 kb EcoRI fragment, oligo B and C recognize a 1.1 kb EcoRI-XhoI fragment. The 3.0 kb EcoRI fragment was subcloned into pUC19 (linearized with EcoRI and dephosphorylated). The 1.1 kb EcoRI-XhoI fragment was subcloned into pUC18 (linearized with EcoRI-SalI, and dephosphorylated). Right subclones were identified by restriction analyses and Southern blot analysis using oligo A or B/C, respectively.

The 3.0 kb EcoRI subclone was called pMT4, the 1.1 kb EcoRI-XhoI subclone was called pMT1. Further restriction analysis of pMT4 and pMT1 was performed using a number of different restriction

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endonucleases (for example: PstI, HindIII and BglII). Southern
blot analysis using oligo A or B/C was carried out to define the
exact region of a possible ORF.

Restriction maps of pDM3, pMT4 and pMT1 are shown on Figure 1.

E.4.2.2. Sequence analysis

From both ends, the DNA inserts of plasmids pMT4 and pMT1 were
sequenced using the universal and reverse primers, priming next
to the polylinker of pUC19/pUC18. Also oligos A, B and C were
used as sequencing primers. The sequencing data resulted in an
ORF of about 400 bp on both sides of the insert of pMT1. Also
pMT4 showed an ORF of about 200 bp when sequenced with the re-
verse primer. Using these sequencing data, an amino acid se-
quence could be deduced. This AA-sequence showed peptide sequen-
ces known from the peptide analysis of the 92 kDa protein
(peptides corresponding to peaks 15, 23, 34 were found). Ac-
cording to these data, the 5'/3' orientation of the gene could
be predicted.

Several other subclones were constructed and sequenced using the
universal and reverse primers of pUC18/19 (Figure 2).

The following oligodeoxynucleotides were also used for sequen-
cing:

Oligo	Sequence (5'-3')	vector sequenced
Oligo 6	CCAACCAGACAACCACTGGG 2713	pMT1
Oligo 7	GACAGGCCACTAACAGCTTC 697	pMT4
Oligo 8	GATGTTGTATGCTGGTGTG 840	pMT4
Oligo 9	CATTGAGCGAGTTGGCAGGG 1178	pMT4
Oligo 4	GAACCTCATGTTTATGAA 2189	pMT1

These oligodeoxynucleotides represent parts of the newly se-
quenced DNA fragments.

For sequencing the 5' region of the gene, exoIII/mung bean dele-
tions of the vector pMT4 were made. pMT4 was linearized using
SphI (3' overlap). The plasmid was then cut using BamHI (5'
overlap).

Exonuclease III deletion was performed according to Roberts and
Lauer (Meth. Enzymol. 68 (1979), 473), Henikoff (Meth. Enzymol.
155 (1987), 156).

Overlapping ends were removed by mung bean nuclease. The resul-
ting plasmids were analyzed by restriction analysis using
HindIII and EcoRI.

Sequence analysis of the clones was carried out using the re-
verse primer of pUC19. The sequence strategy is shown in Figure
3. Sequence data are given in Figure 4.

**Example 5: Northern blot analysis: Identification of mRNA en-
coding Mannosyltransferase.**

E.5.1. Methods

E.5.1.1. Isolation of RNA

Total RNA was isolated from yeast strain SEY2101 (Mat a, ade2-1,
leu2-3, 112, ura3-52 (Emr et al. PNAS 80 (1983), 7080) according
to Domdey et al. (Cell 39 (1984), 611).

E.5.1.2. Northern blot

Total RNA was separated using a formaldehyde agarose gel and
blotted to nitrocellulose as described by Maniatis et al. (loc.
cit.).

E.5.1.3. The DNA-target

The 1.1 kb insert of pMT1 was isolated by EcoRI-PstI digestion. The fragment was purified using the "Gene-clean kit" (Stratagene).

200 ng of the DNA fragment were labeled with $[\alpha\text{-}^{32}P]$-dCTP (50 μCi) using the "megaprime" labeling kit (Amersham) according to the instruction manual of the producer.

E.5.2. Results

The nitrocellulose filter was prehybridized for 2 h at 42°C in 20 ml 5 x Denhardt's, 2 x SSC, 0.1% SDS (w/v), 50% formamide (v/v), 0.1 mg/ml salmon sperm DNA. Hybridization was performed at 42°C for 16 h in 10 ml 1 x Denhardt's, 2 x SSC, 0.1% SDS (w/v), 50% formamide (v/v), 0.1 mg/ml salmon sperm DNA, 200 μg $[\alpha\text{-}^{32}P]$-dCTP labeled 1.1 kb EcoRI-PstI fragment of pMT1. Washing was done twice at RT and two times at 50°C in 50 ml 0.1 x SSC, 0.1% SDS (w/v). Hybridization of the target was detected by exposure to X-ray film (-70°C, 16 h). A single mRNA with the size of 3 kb was detected.   —

This procedure may be easily repeated by the person skilled in the art with other probes derived from the sequence of Figure 4 and with RNA from other sources (other fungal cells).

Example 6: Preparation of an *S. cerevisiae* cell deficient in O-glycosylation activity.

An *S. cerevisiae* cell deficient in O-glycosylation activity was prepared by gene disruption, by insertion of the URA3 gene into the HindIII restriction site of the identified ORF, at bp 1595 of the coding sequence.

E.6.1. Construction of the plasmid used for the gene disruption

The 1.1 kb insert of pMT1 was isolated as EcoRI-PstI fragment and subcloned into a pUC18 vector (EcoRI/PstI linearized, dephosphorylated, without HindIII restriction site in the polylinker). The resulting vector was called pMT1.1.

pMT1.1 was linearized with HindIII and dephosphorylated. The 1.1 kb HindIII fragment of YEp24 (Julius et al., Cell <u>37</u> (1984), 1075) containing the <u>URA3</u> gene of *S. cerevisiae* was isolated and subcloned into the HindIII linearized, dephosphorylated vector pMT1.1. Clones were identified by restriction analyses and called pMT1.1/URA3 (Figure 5).

pMT1.1/URA3 has 0.24 kb PMT1 coding sequence flanking one side of the <u>URA3</u> gene and 0.86 kb PMT1 coding sequence flanking the other. CsCl-DNA of pMT1.1/URA3 was prepared according to Maniatis et al. (loc. cit.).

E.6.2. Transformation of yeast

40 μg of pMT1.1/URA3 CsCl-DNA was digested with SphI/EcoRI. To check that the digestion was complete, part of the digested DNA was analyzed on a DNA agarose gel. The digest was then phenolized and the DNA precipitated with 98% EtOH (Maniatis et al., loc. cit.). DNA was resolved in 10 μl TE, pH 8.0.

S. cerevisiae strains SEY2101/2102 (Mat a/α, ura3-52, leu2-3, 112 (Emr et al., loc. cit.) and SEY2101 (Mat a, ura3-52, leu2-3, 112, ade2-1) were transformed with 5 μl of the EcoRI/SphI digested vector pMT1.1/URA3 according to the method of Ito et al. (J. Bacteriol. <u>153</u> (1983), 163).

SEY2101/2102 transformants were selected on minimal media + Leu; SEY2101 transformants were selected on minimal media + Leu, + Ade.

After 3-4 days at 30°C, transformants could be picked and plated on the same media for a second time.

E.6.3. Genomic Southern blot of the transformants

Genomic DNA of three haploid transformants and wild-type cells
was isolated as described by Hoffmann and Winston (Gene 57
(1987), 267). 1 μg of the genomic DNA was digested with
XhoI/EcoRI, separated on an agarose gel and blotted to nitrocel-
lulose as described by Maniatis et al. (loc. cit.).

The blot was prehybridized in 20 ml 5 x Denhardt's, 2 x SSC,
0.1% SDS (w/v), 0.1 mg/ml salmon sperm DNA, 50% formamide (w/v)
for 4 h at 42°C.

Hybridization was permitted in 10 ml of the same solution adding
200 ng [α-^{32}P]-dCTP labeled 1.1 kb EcoRI/PstI fragment of pMT1.1
(see: E.5.1.3) for 16 h at 42°C. Washing was done two times at
RT in 50 ml 2 x SSC, 0.1% SDS (w/v) and two times at 68°C in 50
ml 1 x SSC, 0.1% SDS (w/v). Signal detection by X-ray films.
Wild-type cells showed a single signal at 1.1 kb, reflecting the
EcoRI/XhoI fragment without URA3 insertion. In the disrupted
strains this signal was missing. Instead of this, a new 2.2 kb
fragment was recognized by the 1.1 kb target, representing the
1.1 kb EcoRI/XhoI fragment bearing the 1.1 kb URA3 insertion.

Example 7: Characterization of the mutant

E.7.1. Growth

SEY2101 wild-type cells were grown either on YPD (10 g/l yeast
extract; 10 g/l peptone; 20 g/l dextrose) or on minimal media +
Ade, + Leu, + Ura. SEY2101 PMT1::URA3 mutant cells were grown
either on YPD or on minimal media + Ade, + Leu. Cells were grown
at 30°C in a waterbath shaker. OD578 was measured every 30' af-
ter sonifying cells. Wild-type and mutant cells show nearly
identical growth on both media although, in some cases, mutant
cells may stick together. Nevertheless, such cells can easily be
separated by sonifying (30", sonify water bath). The growth cha-
racteristics of these cells are listed below:

- Generation time:
    - WT : 99'
    - MT : 93'
- Cell number:
    - WT : 1 OD = 1.9 x 10^7
    - MT : 1 OD = 1.9 x 10^7
- Doubling rate:
    - WT: 0.61/h
    - MT: 0.65/h

In a logarithmically growing culture, 54.7% of wild-type cells and 56% of mutant cells show buds. After growing for 24 h on YPD wild-type cells reached an OD578 of 11.4 and mutant cells of 12.3.

E.7.2. *In vitro* mannosyltransferase activity and Western blot

E.7.2.1. Preparation of crude membranes

SEY2101 was grown in 100 ml minimal media + Ade + Leu + Ura to OD578 = 0.5. SEY2101 PMT1::URA3 was grown in 100 ml minimal media + Ade, + Leu to OD578 = 0.5.

Two preparations of each strain were carried out. Work was performed on ice; all buffers were at 4°C. 40 OD of cells were pelleted and washed in 25 ml TMA (50 mM Tris/HCl, pH 7.5, 7.5 mM MgCl$_2$). Cells were resuspended in 100 μl TMA and transferred to a violax tube. 0.3 g of glass beads were added and cells broken on a vortex four times for 30" (cooling on ice between breaking intervals). The extract is removed from glass beads using a pasteur pipette. Glass beads are washed three times with 250 μl TMA. All washing solutions are pooled in an Eppendorf cup. The solution is centrifuged for 15" (10,000 x g). Supernatant is removed and the pellet resuspended in 40 μl TMA (1 OD = 1 μl)

E.7.2.2. Mannosyltransferase assay (*in vitro*)

1 and 5 μl of the crude membranes (E.7.2.1) were tested for enzyme activity as described by Strahl-Bolsinger and Tanner (loc. cit.). Two parallel samples from wild-type and mutant cells were measured. Mean values of these two independent measurements are shown.

	μl membranes	cpm/incubation[1])
WT	1	2786
WT	5	10289
MT	1	563
MT	5	1135

[1]) Control values (without peptide) were 506 and 1031, respectively.

In contrast to wild-type cells, mutant cells show no *in vitro* mannosyltransferase activity.

E.7.2.3. Western blot analysis

Membranes (1 μl of E.7.2.1) were incubated in 20 μl SDS sample buffer for 1 h at RT. Then SDS/Page and western blot were performed as described by Strahl-Bolsinger and Tanner (loc. cit.). For antibody detection the Peroxidase ECL kit (Amersham) was used according to the instruction manual of the producer. Antibodies against the 92 kDa protein react specifically with a 92 kDa protein of wild-type membranes. In mutant membranes this 92 kDa signal is missing.

E.7.3. *In vivo O-glycosylation*

To investigate *in vivo* glycosylation, wild-type and mutant cells were grown in the presence of [³H]-mannose. Then a crude cell wall plus membrane fraction was isolated and O-glycosylated material released by ß-elimination.

E.7.3.1 Treatment with [³H]-mannose

Wild-type and mutant cells were grown over night in minimal media containing sucrose as only C-source. 7.5 OD of the culture (OD578 = 1-2) were pelleted and washed with 5 ml H_2O (prewarmed 30°C). The cells were grown in 5 ml YP/0.5% sucrose/250 μCi [³H]-mannose in a waterbath shaker for 2 h at 30°C.

E.7.3.2. Isolation of crude cell wall and membrane fraction

5 OD of the [³H]-mannose treated cells were centrifuged and washed three times with 1 ml TMA. Cells were resuspended in 200 μl TMA and broken with glass beads as described in E.7.2.1 (10

μl sample was used for counting radioactivity corresponding to total incorporation). The extract was then centrifuged for 15' (10,000 x g) and the supernatant was removed (100 μl sample was used for counting radioactivity corresponding to soluble material).

E.7.3.3. ß-elimination

The pellet was resuspended in 1 ml 0.1 N NaOH (a 10 μl sample was used for counting radioactivity corresponding to material before ß-elimination). Incubation was maintained for 24 h at 30°C.

E.7.3.4. Analysis of ß-eliminated material

ß-eliminated material was desalted via a Dowex 50WS8/H^+ column (0.5cm x 6cm). The column was saturated with 0.5M mannose and equilibrated in H_2O. The ß-elimination sample was loaded onto the column and washed through with 1.5 μl H_2O. The flow through was collected (a 100 μl sample was used for counting radioactivity corresponding to ß-eliminated material) and concentrated to 10 μl in the speed-vac. Thin-layer chromatography on Silicagel 60 (Merck) in acetone:butanol:H_2O 70:15:15 was performed. Standards: mannose, sucrose, stachyose, raffinose. The chromatographic run was repeated once. Sugars were detected with 0.5 g $KMnO_4$ in 100 ml 1N NaOH. Radioactivity was detected by a thin-layer scanner (Berthold) (see Figure 6).

Cell/ cpm	Total incorporation	Soluble material	Material before ß-elimination	ß-eliminated radioactivity
WT	1.46×10^7	2.23×10^6	1.2×10^7	1.2×10^6
MT	1.12×10^7	1.84×10^6	0.79×10^7	4.0×10^5

Mutant cells show reduced glycosylation in comparison to wild-type cells. The O-glycosylation in mutant cells is about 40-50% lower than in wild-type cells.

Example 8: Cloning of the PMT1 homolog of _Kluyveromyces lactis_.

The yeast _S. cerevisiae_ used to be the system of choice when the production of a heterologous protein in a fungal host cell was desired. However, recent years have shown that the productivity of bakers yeast is often limited, especially when the secretion of the product to the culture medium is required, The use of fungal systems other than _S. cerevisiae_ is therefore preferable in a number of cases [c.f. Romanos et al., Yeast <u>8</u> (1992) 423-488; Fleer, Curr. Opinion Biotechnol. <u>3</u> (1992) 486-496]. One of the alternative yeast hosts is represented by the genus Kluyveromyces for which superior secretion yields have been found with respect to several proteins of commercial interest [e.g. Van den Berg et al., Bio/Technology <u>8</u> (1990) 135-139]. The following example demonstrates that the present invention is not limited to bakers yeast, since the sequence of the PMT1 gene isolated from _S. cerevisiae_ can be advantageously used for the identification of genes encoding similar enzymatic activities in other fungal species. Furthermore, the sequence information revealed in the present invention may also be used for the identification of related mannosyltransferase encoding genes in _S. cerevisiae_.

E.8.1. Design of degenerate PCR primers for the amplification of PMT1 related genes

The region of the PMT1 nucleotide sequence corresponding to the central, hydrophilic region of the mannosyltransferase protein was chosen to design PCR primers [<u>P</u>olymerase-catalyzed <u>C</u>hain <u>R</u>eaction, Saiki et al., Science <u>230</u>(1985) 1350-1354; Mullis & Faloona, Meth. Enzymol. <u>155</u> (1987) 335-350] for the amplification of homologous genes. Amplification requires hybridization, also termed annealing, of these synthetic oligonucleotides with its target DNA. The specificity with which individual regions of genomic DNA are amplified depends on the conditions of the PCR reaction and the degree of homology between the primers and the nucleotide sequence to be amplified. Subsequent to the annealing step, the primers are extended using a thermostable DNA polymerase. Once the complementary strand has been polymerized,

the two strands are separated by heat denaturation and a new
cycle of annealing and polymerization may begin.

Four examples of oligonucleotides suitable as primers for PCR
amplification of PMT1 homologs are presented in Table 2 below.

Table 2

Primer Designation	Nucleotide Sequence
Sq3908	5'ATGGAYGCNAAYAAYGAYTGG-3'
Sq3909	5'-GAYGCNAAYGAYGAYTGGGT-3'
Sq3910	5'-TCYTGYTGYTCRAANCCCCA-3'
Sq3911	5'-CTRTTRTTYTCNCCCCARTA-3'

The design of these "degenerate" oligonucleotides takes into
account that several codons may contain the information for the
incorporation of the same amino acid into a nascent polypeptide
chain, most often varying in the third ("wobble") position of a
triplet. Each primer therefore represents a mixture of oligo-
nucleotides where Y signifies C or T, R signifies A or G, and N
signifies A, C, G or T.

E.8.2. PCR amplification of *K. lactis* genomic DNA

Genomic DNA of *K lactis* strain CBS2359 was prepared as described
by Sherman et al. ["Methods in Yeast Genetics", Cold Spring
Harbor Laboratory Press (1986) p 127]. 10ng of genomic DNA were
used in a standard PCR reaction [Sambrock et al., "Molecular
Cloning - A Laboratory Manual", second edition, Cold Spring
Harbor Laboratory Press (1989)] in the presence of 1 μg of each
of the primers and 5% deionized formamide. The amplification was
performed using a "DNA Thermal Cycler" (Perkin Elmer Cetus) and
"AmpliTag DNA Polymerase" (Perkin Elmer Cetus, 5 units per
reaction tube). The conditions for denaturation, annealing, and
polymerization (30 cycles) were 91°C (1 min), 42°C (2 min), and
72°C (3 min), respectively, except for the first cycle where

denaturation was for 5 min. The results of the PCR amplifica-
tions using the primers disclosed above are presented in Table 3
below.

Table 3

Primer Combinations	Approx. size of amplified DNA DNA fragments (bp)	
	expected for PMT1 homolog	observed
Sq3908+Sq3910	400	400
Sq3908+Sq3911	600	600
Sq3909+Sq3910	170 300	170
Sq3909+Sq3911	400 800	400

These results show that it is not only possible to obtain
fragments exhibiting the same size as that expected for the *K.
lactis* homolog of the *S. cerevisiae* PMT1 gene but that, in
addition, DNA fragments can be amplified with high specificity
that most likely correspond to another gene coding for a closely
related enzymatic activity.

E.8.3 Partial sequence characterization of the *K. lactis* PMT1 homolog

The 400 bp fragment amplified with the primer combination
Sq3908+Sq3910 was subcloned into the vector pCRII (TA Cloning™,
Invitrogen Corp.) following the indications of the supplier, and
partially sequenced according to the method described in
E.4.1.9. using the universal primer. The sequence obtained is
presented in Figure 7. Sequence comparison between the *S.
cerevisiae* PMT1 gene and the fragment isolated from *K. lactis*
genomic DNA by PCR amplification reveals 75% and 80.5% identity
on the nucleotide and amino acid level, respectively (Figure 8).
The amplified 400 bp DNA fragment may be used to target a
selectable marker gene to the *K. lactis* PMT1 chromosomal locus

in analogy to the experiment described under E.6. leading to a disrupted gene. This will yield a *K. lactis* strain with reduced Ser/Thr specific mannosyltransferase activity. In addition, the amplified fragment may be used as homologous hybridization probe for the cloning of the entire *K. lactis* PMT1 gene using standard procedures.

CLAIMS

1. Fungal cells carrying genetic modification(s) within their DNA sequences which cause them to have at least a reduced capacity of O-glycosylation.

2. Fungal cell according to claim 1 wherein said modification(s) comprise any suppression, substitution, deletion, addition, disruption and/or mutational insertion.

3. Fungal cell according to claim 2 wherein said modification(s) are stable during segregation and/or non-reverting and/or non-leaky.

4. Fungal cell according to any of claims 1 to 3 wherein said modification(s) are located in one or more coding regions of the DNA sequences of the cell.

5. Fungal cell according to any of claims 1 to 3 wherein said modification(s) are located in one or more regions responsible for or involved in the expression and/or transcriptional regulation of a gene.

6. Fungal cell according to claim 4 or 5 wherein said gene is a gene whose expression product is an enzyme of the O-glycosylation pathway.

7. Fungal cell according to claim 6 wherein the reduced capacity of O-glycosylation results from the production of inactive enzymes, from the production of enzymes having altered biological properties, from the absence of production of said enzymes, or from the production of said enzymes at low levels.

8. Fungal cell according to claim 6 wherein said gene is a gene whose expression product is involved in the attachment of mannosyl residues to the hydroxyl group of seryl or threonyl amino acids.

9.   Fungal cell according to claim 8 wherein said gene is a
     gene whose expression product is involved in the transfer
     of mannosyl residues from the Dol-P-Man donor to the hy-
     droxyl group of seryl or threonyl amino acids.

10. Fungal cell according to claim 9 wherein said gene is the
     gene encoding the Dol-P-Man:Protein (Ser/Thr) Mannosyl
     Transferase [PMT1] whose sequence is represented in Figure
     4, or any homologous gene encoding the same activity.

11. Fungal cell according to any of claims 8 to 10, further
     comprising modification(s) in one or more genes involved in
     subsequent additions of mannosyl residues, or in the syn-
     thesis of the mannosyl residues donor (Dol-P-Man).

12. Fungal cell according to any of the preceding claims in
     which an exogenous DNA sequence has been introduced.

13. Fungal cell according to claim 12 wherein the exogenous DNA
     sequence comprises one or more genes encoding a desired
     protein to be expressed and/or secreted in said cell.

14. Fungal cell according to claim 13 wherein said DNA sequence
     is included in an expression cassette comprising a tran-
     scription and translation initiation region joined to the
     5' end of said DNA sequence encoding the desired pro-
     tein(s).

15. Fungal cell according to claim 14 wherein said transcrip-
     tion and translation initiation region is chosen from pro-
     moters derived from fungal cell genes.

16. Fungal cell according to claim 14 wherein said expression
     cassette further comprises a transcription and translation
     termination region at the 3' end of the DNA sequence en-
     coding the desired protein(s).

17. Fungal cell according to claim 14 wherein said expression cassette further comprises a signal peptide (pre-sequence) at the N-terminus of the desired protein sequence so as to direct the nascent protein to the secretory pathway of said fungal cell.

18. Fungal cell according to any of claims 12 to 17 wherein the exogenous DNA sequence is part of a vector which may either replicate autonomously in said fungal cell or integrate into its own DNA sequences (chromosome).

19. Fungal cell according to any of the preceding claims wherein it is chosen from filamentous fungi and yeasts.

20. Fungal cell according to claim 19 wherein the filamentous fungi is chosen from the group consisting of *Aspergillus, Trichoderma, Mucor, Neurospora, Fusarium,* and the like.

21. Fungal cell according to claim 19 wherein the yeast is chosen from the group consisting of *Kluyveromyces, Saccharomyces, Pichia, Hansenula, Candida, Schizosaccharomyces* and the like.

22. Fungal cell according to claim 21 wherein the yeast is chosen from the group consisting of *Kluyveromyces, Saccharomyces, Pichia, Hansenula,* and *Candida,* and, more preferably, from *Kluyveromyces* and *Saccharomyces.*

23. Process for the production of recombinant products wherein a fungal cell according to claims 12-22 is cultivated in conditions in which the exogenous DNA sequence is expressed, and the product is recovered.

24. Process according to claim 23 wherein said product is secreted into the culture medium.

25. Process according to claims 23 or 24 wherein said product is susceptible to O-glycosylation by the fungal cell.

26. DNA fragment encoding an enzyme involved in the attachment of mannosyl residues to the hydroxyl group of seryl or threonyl amino acids of proteins.

27. DNA fragment according to claim 26 comprising the Dol-P-Man:Protein (Ser/Thr) Mannosyl Transferase gene whose sequence is represented in Figure 4, or any fragment or derivative thereof.

28. DNA fragment according to claim 26 comprising all or part of the sequence presented in Figure 7.

29. The use of a DNA fragment according to claim 26 or 27 or any part thereof as hybridization probe(s) or for the complementation of mutant phenotypes for the obtention of homologous genes of fungal cells.

30. DNA fragments Sq3908, Sq3909, Sq3910 and Sq3911.

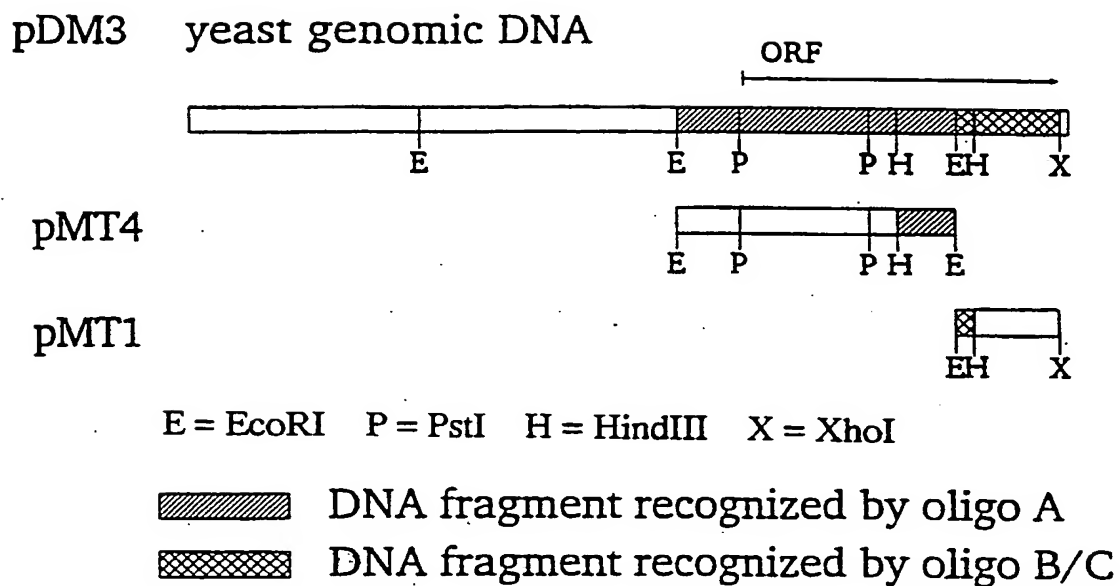pDM3　　yeast genomic DNA
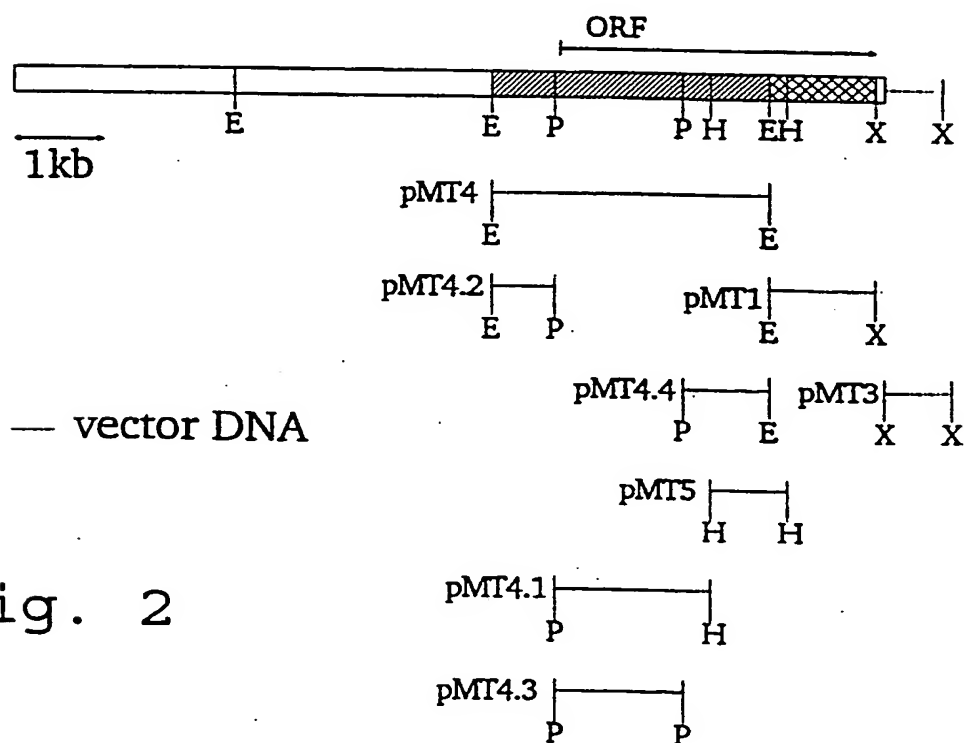

Fig. 1

Fig. 2

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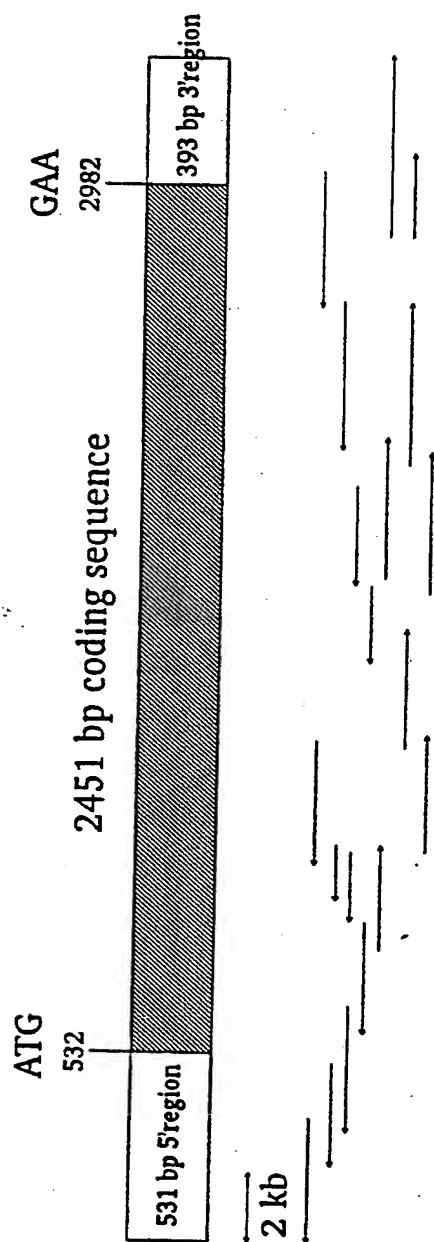
Fig. 3

Fig.4A

SEQ ID NO :1

TYPE OF SEQUENCE: Nucleotides
LENGTH: 3375 base pairs
NUMBER OF STRANDS: 1
CONFIGURATION: Linear
TYPE OF MOLECULE: cDNA
ORIGIN :   Saccharomyces cerevisiae


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  1   GCTTGTCTCC TGTTCATTCT ACGCTTGTCA TCCACACTGG CACCGGCAGC
 51   GGCGCTGCTC TGTGAGCTGT CTTTATCCAG CAGGCTCAAA TAGACTTGTT
101   CTGCCTCATA TACTGTTTTT CATTAACCAG TCTCCTGGCC TCTTCTAATT
151   TCGAACCTGG CAGAGACATG AAGTTCCTCG TTATAGATTA ATCACCAATT
201   GTCCTCTTTC AGCGCTTCTG TTAGTTGGCC TTATTTAACG GATCTTTGCT
251   TCAATTACGC TTTCATCCAA CATTTGCCAC CCTTGGAACC AGAAGAGAAT
301   ATACATCATT CGGGGTTGCC CTGCCCATTT ATATCGTATA TTGTGATGAT
351   ATACCTTTTT TTTTTTTTTC GCTGCGTTTT TTTTCTCGAC ACGTGTCGAA
401   GAAGAGTTTG GCGTTTCTAA GCAGATCTTG ATTATTTTCG AGCAGCAAAA
451   CAAGACAAAC AGGTGCATTG TTAAAGCGAG GTAGTATCAG AAGAGCCTAT
501   CAAGAAACAG CTAACAGCTA CAAGCACGGT CATGTCGGAA GAGAAAACGT
551   ACAAACGTGT AGAGCAGGAT GATCCCGTGC CGAACTGGA TATCAAGCAG
601   GGCCCCGTAA GACCCTTTAT TGTTACCGAT CCGAGTGCCG AATTGGCCTC
651   GTTACGAACC ATGGTCACTC TTAAAGAGAA GCTGTTAGTG GCCTGTCTTG
701   CTGTCTTTAC AGCGGTCATT AGATTGCATG GCTTGGCATG GCCTGACAGC
751   GTGGTGTTTG ATGAAGTACA TTTCGGTGGG TTTGCCTCGC AATACATTAG
801   GGGGACTTAC TTCATGGATG TGCATCCTCC TCTTGCAAAG ATGTTGTATG
851   CTGGTGTGGC ATCGCTTGGT GGGTTCCAGG GTGATTTTGA CTTCGAAAAT
901   ATTGGTGACA GCTTTCCATC TACGACGCCA TACGTGTTGA TGAGATTTTT
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1001  GTTATTCTGG  TGTTCGTATG  TGGGTTGCTT  TGATGAGCGC  TATCTGCTTT
1051  GCCGTTGAAA  ACTCGTACGT  CACTATTTCT  CGTTACATTC  TGTTGGACGC
1101  CCCATTGATG  TTTTTCATTG  CAGCTGCAGT  CTACTCTTTC  AAGAAATACG
1151  AAATGTACCC  TGCCAACTCG  CTCAATGCTT  ACAAGTCCTT  GCTTGCTACT
1201  GGTATTGCTC  TTGGTATGGC  ATCTTCATCC  AAATGGGTTG  GTCTTTTCAC
1251  GGTTACATGG  GTGGGTCTTT  TATGTATCTG  GAGACTATGG  TTCATGATTG
1301  GGGATTTGAC  TAAGTCTTCC  AAGTCCATCT  TCAAAGTAGC  ATTTGCCAAA
1351  TTGGCCTTCT  TGTTGGGTGT  GCCTTTTGCC  CTTTATCTGG  TCTTCTTTTA
1401  TATCCACTTC  CAATCATTAA  CTTTGGACGG  GGATGGCGCA  AGCTTCTTTT
1451  CGCCTGAATT  TAGATCTACA  CTAAAGAACA  ATAAGATCCC  CCAAAATGTC
1501  GTTGCTGATG  TCGGCATTGG  CTCCATTATC  AGCTTGCGTC  ATCTCTCTAC
1551  CATGGGCGGT  TATTTGCATT  CTCATTCACA  CAATTATCCA  GCTGGTTCGG
1601  AACAACAACA  AAGCACTTTA  TATCCTCACA  TGGATGCCAA  TAACGATTGG
1651  TTGTTGGAAC  TTTACAACGC  ACCCGGCGAA  TCTTTAACAA  CATTCCAAAA
1701  CCTAACCGAT  GGTACCAAGG  TCAGACTATT  CCACACTGTT  ACAAGATGTA
1751  GATTACACTC  TCATGACCAT  AAGCCACCCG  TTTCAGAAAG  CAGCGACTGG
1801  CAGAAGGAGG  TTTCTTGTTA  TGGTTACAGC  GGATTCGACG  GTGATGCTAA
1851  TGATGACTGG  GTTGTTGAGA  TTGATAAAAA  GAATTCTGCT  CCTGGAGTTG
1901  CCCAAGAACG  GGTCATAGCT  TTGGACACAA  AGTTTAGATT  GAGACATGCT
1951  ATGACAGGCT  GTTATTTGTT  TTCCCACGAA  GTCAAGTTGC  CAGCTTGGGG
2001  GTTCGAACAA  CAAGAAGTTA  CCTGTGCCTC  CTCCGGTAGA  CATGATTTAA
2051  CATTGTGGTA  CGTTGAGAAC  AACAGTAACC  CATTGTTACC  AGAAGATACC
2101  AAGCGTATTT  CCTATAAACC  TGCAAGCTTC  ATTTCTAAAT  TTATTGAATC
2151  CCATAAAAAG  ATGTGGCATA  TCAATAAAAA  TTTGGTCGAA  CCTCATGTTT
2201  ATGAATCACA  ACCAACTTCA  TGGCCATTCT  TGCTACGTGG  TATAAGTTAC
2251  TGGGGTGAAA  ATAACAGAAA  CGTCTATCTA  TTAGGTAATG  CGATCGTATG
2301  GTGGGCTGTC  ACCGCTTTCA  TCGGTATTTT  CGGATTGATT  GTTATCACTG
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SUBSTITUTE SHEET

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2351 AGCTGTTCTC GTGGCAGTTA GGTAAACCAA TTTTGAAGGA CTCCAAGGTT
2401 GTTAACTTCC ACGTTCAGGT TATTCACTAC TTATTGGGTT TTGCCGTCCA
2451 TTATGCTCCA TCTTTCTTAA TGCAACGTCA AATGTTTTTG CATCACTACT
2501 TACCTGCTTA TTATTTCGGT ATTCTTGCCC TTGGCCACGC CTTGGACATA
2551 ATAGTTTCTT ATGTTTTCCG CAGCAAGAGA CAAATGGGCT ACGCGGTAGT
2601 GATCACTTTC CTTGCTGCTT CTGTGTATTT CTTCAAGAGC TTCAGTCCAA
2651 TTATTTACGG TACACCATGG ACTCAAGAAT TGTGTCAAAA ATCGCAGTGG
2701 TTGTCTGGTT GGGACTACAA TTGTAACACA TACTTTTCTT CATTAGAAGA
2751 GTACAAAAAC CAAACCTTGA CTAAACGTGA ATCTCAACCT GCCGCCACTA
2801 GTACAGTTGA AGAAATCACT ATAGAAGGGG ACGGTCCGTC GTATGAAGAT
2851 CTCATGAACG AGGATGGCAA GAAAATCTTT AAAGACACAG AAGGTAATGA
2901 ACTAGATCCA GAAGTTGTCA AAAAAATGTT GGAAGAGGAG GGAGCTAACA
2951 TTTTAAAAGT AGAAAAAAGG GCTGTTTTGG AATAAATTTC CCAGTACTCT
3001 CCACATTTTT ATGTAAAGTC TTCTATAAGC TCTCGAGCGT ATAATTAAAA
3051 ACGAAAATAG ACAAAAAAAA CATCATGAAT AAAAAAAATG TCTTGAAGCT
3101 GACTATATTG TCCATCTGCG TTTAGAGACA CGTATTCTAT TTCGCTCAAA
3151 TAAGTATGAT CTGCAAGTAG TTTCAGTGGT ATTATCATTT CGCACCGTTT
3201 TTTTTCCAAG AACTCGTTTA CGTGCCGCGA AAAGTCTATC GAATAGGCAT
3251 TCGAGAACAA TAGAAAGGA ACAGAAGCGT AGTACATATT ATGCATAGAC
3301 CCGTTTCTTT TCTTCTTTTT CGAAAATATT CTTATTGATT TAACAATTAA
3351 GCAGGTGTGT AAGATCAGAA CTGCA
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SUBSTITUTE SHEET

SEQ ID NO: 2

TYPE OF SEQUENCE: Peptidic
LENGTH :   817 amino acids
PROPERTY: Dol-P-Man:protein(Ser/Thr)Mannosyltransferase
ORIGIN: Saccharomyces cerevisiae


```
  1   MSEEKTYKRV EQDDPVPELD IKQGPVRPFI VTDPSAELAS LRTMVTLKEK
 51   LLVACLAVFT AVIRLHGLAW PDSVVFDEVH FGGFASQYIR GTYFMDVHPP
101   LAKMLYAGVA SLGGFQGDFD FENIGDSFPS TTPYVLMRFF SASLGALTVI
151   LMYMTLRYSG VRMWVALMSA ICFAVENSYV TISRYILLDA PLMFFIAAAV
201   YSFKKYEMYP ANSLNAYKSL LATGIALGMA SSSKWVGLFT VTWVGLLCIW
251   RLWFMIGDLT KSSKSIFKVA FAKLAFLLGV PFALYLVFFY IHFQSLTLDG
301   DGASFFSPEF RSTLKNNKIP QNVVADVGIG SIISLRHLST MGGYLHSHSH
351   NYPAGSEQQQ STLYPHMDAN NDWLLELYNA PGESLTTFQN LTDGTKVRLF
401   HTVTRCRLHS HDHKPPVSES SDWQKEVSCY GYSGFDGDAN DDWVVEIDKK
451   NSAPGVAQER VIALDTKFRL RHAMTGCYLF SHEVKLPAWG FEQQEVTCAS
501   SGRHDLTLWY VENNSNPLLP EDTKRISYKP ASFISKFIES HKKMWHINKN
551   LVEPHVYESQ PTSWPFLLRG ISYWGENNRN VYLLGNAIVW WAVTAFIGIF
601   GLIVITELFS WQLGKPILKD SKVVNFHVQV IHYLLGFAVH YAPSFLMQRQ
651   MFLHHYLPAY YFGILALGHA LDIIVSYVFR SKRQMGYAVV ITFLAASVYF
701   FKSFSPIIYG TPWTQELCQK SQWLSGWDYN CNTYFSSLEE YKNQTLTKRE
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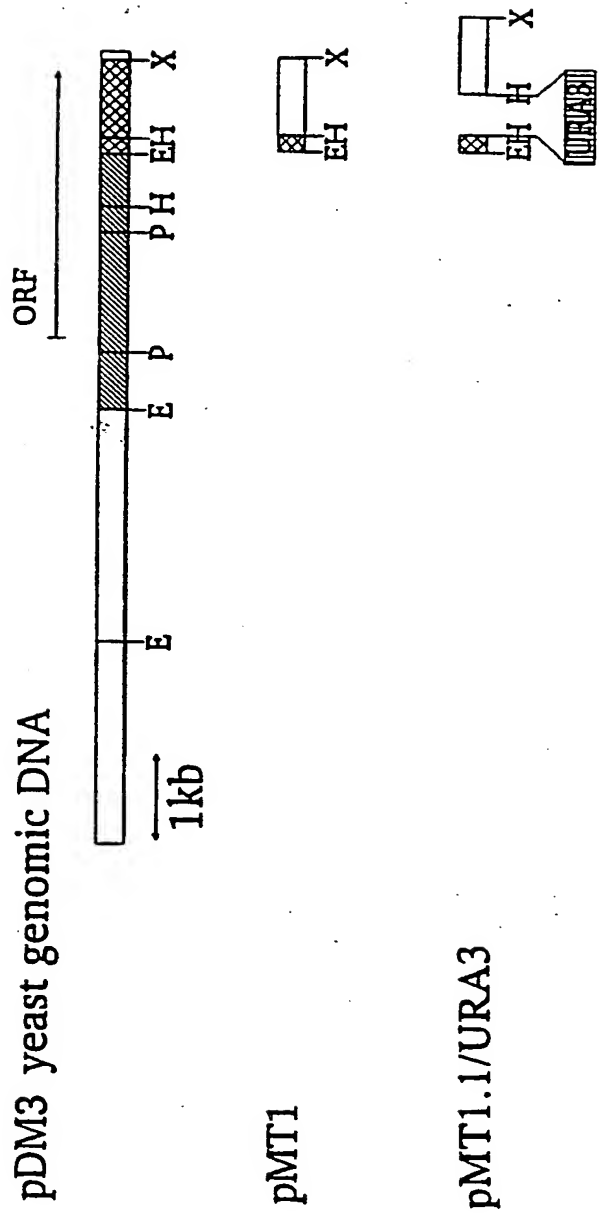
Fig.4B

Fig. 5

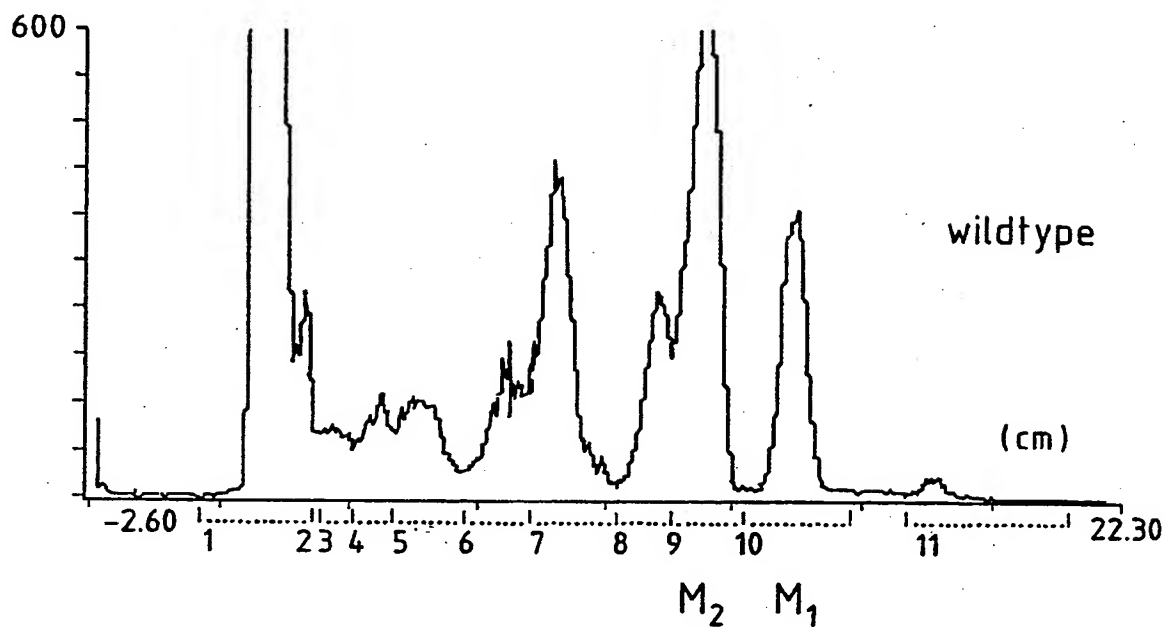TITLE: WT Eliminie LIN
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M₂ M₁

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C -2.35 6 ROI

mutant

(cm)

M₂ M₁
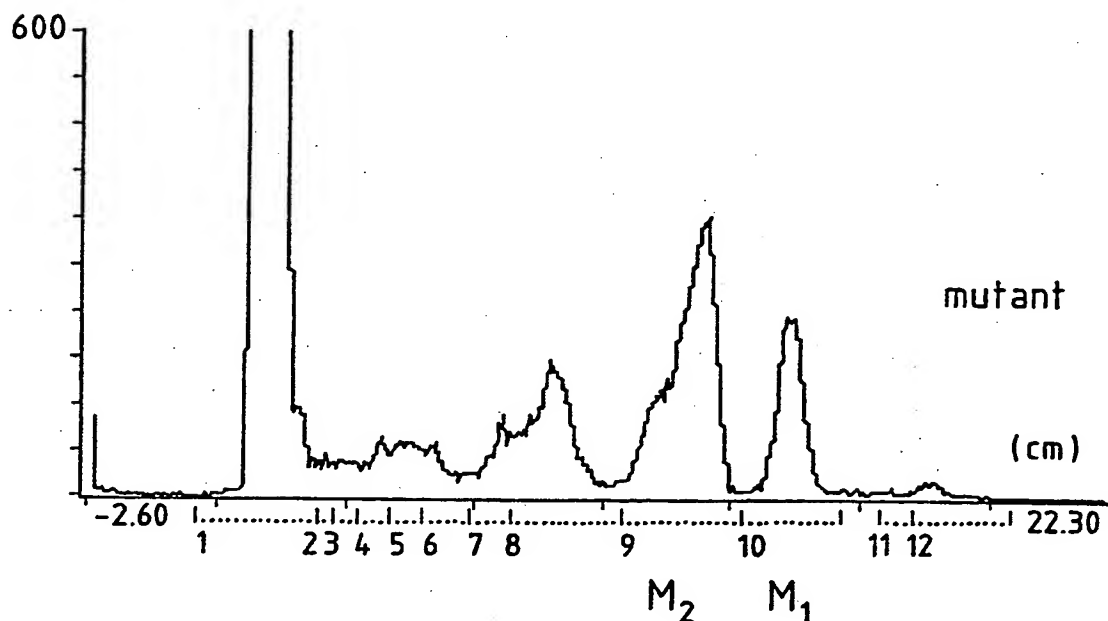
Fig. 6

SEQ ID No. 3

Sequence Type:	DNA
Sequence Length:	126 base pairs
Topology:	linear
Strandedness:	single
Feature:	part of the K. lactis Dol-P-Man:protein (Ser/Thr) mannosyltransferase encoding gene (open reading frame)
Origin:	Kluyveromyces lactis

```
  1 TCTGCTCCTG GCGNAGCTCA AGTATNCGTT AAGGCTTTGG ACACTAAATT
 51 CAGATTGAGA CATGCTATGA CTGGTTGTAG TATCTCACAT GAAGTCAAAT
101 TACCAAAATG GGGCTTCGAA CAACAG
```

Fig.7

(A)

position 1885 of SEQ. ID N° 1

TCTGCTCCT GGAGTTGCC CAAGAACGG GTCATAGCT TTGGACACA AAGTTTAGA
....
TCTGCTCCT GGCG?AGCT CAAGTAT?C GTTAAGGCT TTGGACACT AAATTCAGA

TTGAGACAT GCTATGACA GGCTGTTAT TTGTTTTCC CACGAAGTC AAGTTGCCA
....
TTGAGACAT GCTATGACT GGTTGTAGT ATC---TCA CATGAAGTC AAATTACCA

GCTTGGGGG TTCGAACAA CAA
....
AAATGGGGC TTCGAACAA CAG

(B)

position 452 of SEQ. ID N° 2

SAPGVAQER VIALDTKFR LRHAMTGCY LFSHEVKLP AWGFEQQ
....
SAPG?AQV? VKALDTKFR LRHAMTGCS I-SHEVKLP KWGFEQQ

Fig.8

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5   C12N15/54   C12N9/10   C12N15/81   C12N1/15   C12N1/19
         C12P21/00   C12Q1/68   //(C12N1/19,C12R1:865)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5   C12N   C12P   C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EUR. J. BIOCHEM. vol. 196, no. 1 , February 1991 , SPRINGER VERLAG, BERLIN, BRD; pages 185 - 190 S. STRAHL-BOLSINGER AND W. TANNER 'Protein O-glycosylation in Saccharomyces cerevisae' cited in the application see page 189, right column, line 26 - page 190, left column, line 2 ---	1-30
Y	US,A,4 929 553 (BUSSEY ET AL.) 29 May 1990 see column 3, line 60 - column 4, line 8 see column 6, line 6 - line 31 --- -/--	1-30

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 1993	18 -01- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FEBS LETTERS vol. 248, no. 1,2 , 8 May 1989 , ELSEVIER PUBLISHERS, AMSTERDAM, NL; pages 111 - 114 K. HARD ET AL. 'O-mannosylation of recombinant human insulin-like growth factor I (IGF-I) produced in Saccharomyces cerevisiae' cited in the application the whole document	1-30
A	METHODS IN ENZYMOLOGY vol. 194 , 1991 , ACADEMIC PRESS, INC., NEW YORK, US; pages 281 - 301 R. ROTHSTEIN 'Targeting, disruption, replacement, and allele rescue: Integrative DNA transformation in yeast' cited in the application see page 290, line 9 - page 298, line 17	1-30
A	BIO/TECHNOLOGY vol. 8, no. 2 , February 1990 , NATURE AMERICA, INC., NEW YORK, US; pages 135 - 139 J.A. VAN DEN BERG ET AL. 'Kluveromyces as a host for heterologous gene expression: Expression and secretion of prochymosin' cited in the application the whole document	1-30